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# A Myomodulin-CARP-related Peptide Isolated from a Polychaete Annelid, *Perinereis vancaurica*

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**ABSTRACT**—Myomodulin-CARP-family peptides have been isolated only from molluscs. In the present study, a heptapeptide, Ala-Met-Gly-Met-Leu-Arg-Met-$NH_2$, termed Pev-myomodulin, was isolated from a polychaete annelid, *Perinereis vancaurica* using the esophagus of the animal as the bioassay system. The sequence of the annelid peptide is highly homologous with those of the myomodulin-CARP-family peptides found in molluscs. The annelid peptide is regarded as a member of the myomodulin-CARP family, though all the molluscan peptides have a Leu-$NH_2$ at their C-termini. The annelid peptide showed a potnet contractile action on the esophagus of the annelid. The peptide may be an excitatory neuromediator involved in the regulation of the esophagus. Among various myomodulin-CARP-family peptides and their analogues, the annelid peptide showed the most potent contractile action on the esophagus. Replacement of the C-terminal Met-$NH_2$ of the annelid peptide with a Leu-$NH_2$ decreased its contractile potency, while replacement of the C-terminal Leu-$NH_2$ of myomodulin and CARP with a Met-$NH_2$ increased their potency. The C-terminal Met-$NH_2$ of the annelid peptide seems to be important, but not essential, for exhibiting its contractile activity on the esophagus. On the anterior byssus retractor muscle of the bivalve mollusc *Mytilus edulis*, the annelid peptide showed catch-relaxing and contraction-modulating effects qualitatively similar to those of the authentic peptide CARP, though the annelid peptide was less potent than CARP.

## INTRODUCTION

Over the past two decades, a large number of bioactive peptides have been isolated from invertebrates, especially from arthropods and molluscs [5, 9, 12, 18, 20. 21]. As to annelids, however, only several FMRFamide-related peptides have been identified using radioimmunoassay for detection of the peptides. Krajniak and Price [13] found FMRFamide in the polychaete *Nereis virens*. Baratte *et al.* [1] isolated FMRFamide and its analogue from *Nereis diversicolor*. Evans *et al.* [4] identified FMRFamide and four analogues in the medicinal leech *Hirudo medicinalis*.

In the previous study, we isolated two S-Iamide-family peptides, AKSGFVRIamide and VSSFVRIamide, from the polychaete annelid, *Perinereis vancaurica* [16]. The peptides show a potent contractile effect on the esophagus of the annelid [16], while FMRFamide shows an inhibitory effect [14]. This was the first finding of annelid peptides which were not considered to be FMRFamide-family peptides. Since this finding, we have continued to search for bioactive peptides in the annelid *P. vancaurica* using its esophagus as the bioassay system. In the present study, we found a novel heptapeptide, Ala-Met-Gly-Met-Leu-Arg-Met-$NH_2$, that showed a potent excitatory action on the esophagus. The sequence of the peptide is highly homologous to myomodulin, catch-relaxing peptide (CARP) and their related peptides, all of which have been isolated from molluscs ([2, 3, 8, 10, 11], See also Table 1). That is, the annelid peptide is regarded as a member of myomodulin-CARP family. Here, we report purification, structure determination and pharmacological characterization of the annelid myomodulin-CARP-family peptide.

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## MATERIALS AND METHODS

### *Animals*

The marine polychaete annelid worms, *Perinereis vancaurica,* were purchased from a fishing-bait store and the sea mussels, *Mytilus edulis,* were collected in the Hiroshima Bay. These animals were kept in laboratory tanks filled with aerated seawater at 15°C.

### *Extraction and purification*

Extraction procedures for bioactive peptides in *P. vancaurica* were essentially the same as those reported previously [16]. Briefly, 0.5 kg of the animals were boiled for 10 min at 100°C in 2 l of water containing 4% acetic acid and homogenized with a Waring blender and then with a Polytron homogenizer. The homogenate was centrifuged at 15,000×*g* for 40 min at 4°C. The supernatant was concentrated with a rotary evaporator, and 1/10 volume of 1 N HCl was added to the concentrated solution with constant agitation. The concentrated solution was centrifuged again. The supernatant was applied to two C-18 cartridges (Mega Bond Elut, Varian) in series. After the cartridges were washed with 0.1% trifluoroacetic acid (TFA), the retained material was eluted with 50% methanol. The

eluate was applied to four steps of reversed-phase and cation-exchange high performance liquid chromatography (HPLC). The eluting substances were monitored with an UV detector at 220 nm. Each of the fractions obtained at each HPLC step was bioassayed using the isolated esophagus of *P. vancaurica*.

At the first HPLC-purification step, the eluate from the C-18 cartridges was applied to a Capcell-Pak C-18 reversed-phase column (Shiseido, 10×250 mm). The column was eluted with a 120-min linear gradient of 0–60% acetonitrile (ACN) in 0.1% TFA (pH 2.2). The flow rate was 1 ml/min. An aliquot (1/250) of each 2-ml fraction was evaporated to dryness, dissolved in artificial seawater (ASW) and subjected to bioassay. A bioactive peak eluted at around 20% ACN was then subjected to the second step of HPLC purification. At this step, another C-18 reversed-phase column (ODS-80TM, Tosoh, 4.6×150 mm) was used. The column was eluted with a 50-min linear gradient of 15–25% ACN in 0.1% TFA. A bioactive peak was observed at around 16% ACN. At the third step, the bioactive substance was applied to a cation-exchange column (SP-5PW, Tosoh, 7.5×75 mm), and the column was eluted with a 70-min linear gradient of 0–0.7 M NaCl in 10 mM phosphate buffer (pH 7.1) at a flow rate of 0.5 ml/min. The bioactive substance was eluted at around 0.22 M NaCl. At the fourth step (final step), the bioactive substance was applied to the reversed-phase column (ODS-80TM), and the column was eluted with a 25-min linear gradient of 15–25% ACN in 0.1% TFA at a flow rate of 0.5 ml/min. The substance was eluted at around 20% ACN as a single absorbance peak (Fig. 1).

The purified bioactive substance was subjected to peptide sequence analysis (Shimadzu PSQ-1 Protein Sequencer) and fast atom bombardment mass spectrometric (FAB-MS) analysis (JEOL JMS-HX 110/110A). These analyses suggested that the substance is a pentapeptide with an amidated C-terminus. The peptide having the suggested structure was then synthesized by a solid-phase peptide synthesizer (Applied Biosystems 430A) and purified by HPLC. The structure of the synthesized peptide was confirmed by amino acid sequence analysis and FAB-MS analysis. The synthetic peptide was compared with the native one in the behavior on HPLC.

*Bioassay*

The anterior half of the body was cut open ventrally and the esophagus (about 15 mm long) was excised. Both ends of the isolated esophagus were ligated with cotton threads. One of the thread was tied to a fixed support of a trough (2 ml) filled with aerated ASW, and the other was connected to a force-displacement transducer attached to a manipulator. Changes in the esophagus tension in response to bioactive substances were recorded on a chart recorder through an amplifier.

Aliquots (1/250–1/100) of the fractions obtained at each HPLC purification step were evaporated to dryness, dissolved in 0.1 ml ASW and injected into the aerated trough in which the isolated esophagus was mounted. After each recording of the effects of the fractions, the esophagus was washed with ASW. The next test was started 10 min after the recording.

*Pharmacology*

The contractile actions of 10 myomodulin-CARP-family peptides including the annelid peptide isolated in the present study and nine synthetic analogues were examined on the isolated esophagus of *P. vancaurica*. It has been shown that CARP powerfully relaxes catch tension in the anterior byssus retractor muscle (ABRM) of *M. edulis* and that the peptide potentiates phasic contraction of the ABRM in response to repetitive electrical stimulation at lower concentrations and inhibits the contraction at higher concentrations [6, 7, 18]. Therefore, the actions of the annelid peptide on catch tension and phasic contraction of the ABRM were also examined to compare them with those of CARP. Catch contraction was produced by applying $10^{-4}$ M acetylcholine (ACh) to the ABRM for 2 min at 20 min intervals and phasic contractions was elicited by stimulating the muscle with repetitive electrical pulses (15 V, 3 msec, 10 Hz, 50 pulses) at 10 min intervals. This procedure was basically the same as that of Muneoka and Twarog [19].

*Saline*

The saline used for the esophagus of *P. vancaurica* and the ABRM of *M. edulis* was ASW of the following composition: 445 mM NaCl, 10 mM KCl, 10 mM $CaCl_2$, 55 mM $MgCl_2$ and 10 mM Tris-HCl (pH 7.6).

## RESULTS

After the three steps of HPLC purification, the final purification was performed with the reversed-phase column (Fig. 1). The single absorbance peak obtained was found to coincide with an active peak. At the first step of HPLC purification on a Capcell-Pak C-18 reversed-phase column, the active substance was recovered in the same fraction (No. 34) as a previously reported S-Iamide peptide, VSSFVRIamide [16]. At the second step of HPLC using another C-18 reversed-phase column (ODS-80TM), these two substances were separated from each other: VSSFVRIamide was recovered in fractions 7, 8, and 9, while the present substance was in fractions 12 and 13.

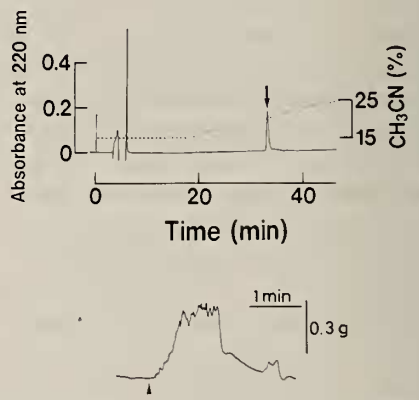


Fig. 1. HPLC profile at the final step of purification of the bioactive substance on the C-18 ODS-80TM column (upper) and the activity of the purified substance on the esophagus of *P. vancaurica* (lower). The elution was performed with a linear gradient of 15–25% ACN (0.1% TFA) at a flow rate of 0.5 ml/min. The absorbance was monitored at 220 nm. The broken line shows the concentration of ACN. An aliquot (1/100) of the purified substance was applied at the time indicated by an arrow head.

The determined sequence and detected amount (picomoles) of each amino acid in the amino acid sequence analysis of the purified substance were as follows: Ala (820.6)-Met

(892.2)-Gly (508.6)-Met (808.2)-Leu (729.2)-Arg (114.3)-Met (293.1). In the FAB-MS spectrum of the purified substance, a molecular ion peak was observed at 810.1 m/z $(M+H)^+$. The results of these analyses suggested that the purified substance is an amidated heptapeptide having the following primary structure: Ala-Met-Gly-Met-Leu-Arg-Met-$NH_2$. The yield of this peptide from 500 g of the worms was roughly estimated to be 3 nmoles using the data on amino acid sequence analysis. The value was comparable to the yield of an S-Iamide peptide, AKSGFVRIamide, and larger than the yield of another S-Iamide peptide, VSSFVRIamide [16].

A mixture of the synthetic peptide with the suggested structure and the native peptide (purified substance) showed a single absorbance peak when applied to a C-18 reversed-phase column and a cation-exchange column (Fig. 2). We were not able to compare the bioactivity between the synthetic and native peptides, because the quantity of the native peptide obtained was not enough for the comparison. However, we confirmed contraction-eliciting activity of the synthetic peptide at concentrations of $10^{-8}$ M or higher (Fig. 3).

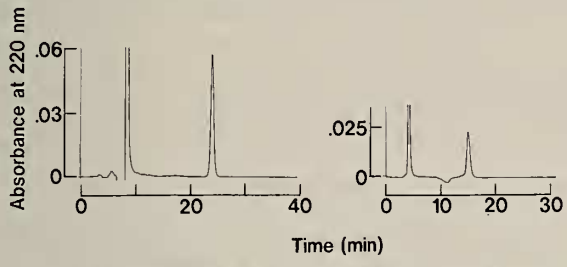


Fig. 2. HPLC profiles of mixtures of the native and synthetic peptides on a C-18 ODS-80TM column with an isocratic elution of 21% ACN (0.1% TFA) at a flow rate of 0.3 ml/min (left) and on the cation-exchange SP-5PW column with an isocratic elution of 0.2 M NaCl (10 mM phosphate buffer, pH 7.1) at a flow rate of 0.5 ml/min (right).

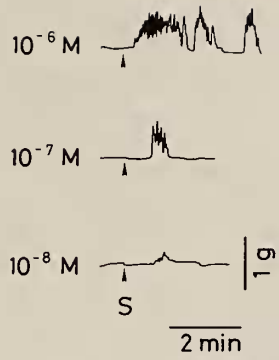


Fig. 3. Actions of the synthetic peptide on the isolated esophagus at different concentrations. The peptide was applied at the times indicated by arrow heads.

The sequence of the annelid peptide is highly homologous to those of myomodulin-CARP-family peptides found in several molluscs (Table 1). We examined the effects of these molluscan peptides and several synthetic analogues on the esophagus of *P. vancaurica,* and compared the effects with those of the annelid peptide which we designated Pev-myomodulin (Table 2).

Pev-myomodulin showed the most potent contractile effect on the esophagus, and was approximately 10 times more potent than myomodulin and CARP. In Figure 4, the effects of $10^{-6}$ M Pev-myomodulin and $10^{-6}$ M CARP on a preparation of the esophagus are shown. $Met^7$-CARP and $Met^7$-myomodulin were also more potent than the respective authentic molluscan peptides, though they were slightly less potent than Pev-myomodulin. $Phe^7$-CARP and $Phe^7$-myomodulin were far less potent than Pev-myomodulin. $Leu^7$-Pev-myomodulin was also far less potent than the authentic annelid peptide. C-terminal-free CARP did not show any effect even at $10^{-5}$ M. Met-Leu-Arg-Leu-$NH_2$, a common C-terminal fragment of the most molluscan myomodulin-CARP-family peptides, showed a considerable effect,

Table 1. Myomodulin-CARP-family peptides

| Phyla | Animals | Structures | References |
|---|---|---|---|
| Annelida | *Perinereis* | AMGMLRMamide | this study |
| Mollusca | *Mytilus* | AMPMLRLamide | Hirata *et al.* (1987) |
| | *Aplysia* | PMSMLRLamide | Cropper *et al.* (1987) |
| | | GSYRMMRLamide | Cropper *et al.* (1991) |
| | *Fusinus* | PMSMLRLamide | Kanda *et al.* (1990) |
| | | PMNMLRLamide | Kanda *et al.* (1990) |
| | *Helix* | PMSMLRLamide | Ikeda *et al.* (1993) |
| | | SLGMLRLamide | Ikeda *et al.* (1993) |
| | | GLNMLRLamide | Ikeda *et al.* (1993) |
| | | pQLSMLRLamide | Ikeda *et al.* (1993) |
| | | pQLPMLRLamide | Ikeda *et al.* (1993) |
| | *Achatina* | SLGMLRLamide | Ikeda *et al.* (unpublished) |
| | | GLHMLRLamide | Ikeda *et al.* (unpublished) |

pQ, pyroglutamic acid.

Table 2. Contractile effects of myomodulin-CARP-family peptides and synthetic analogues on the isolated esophagus of *Perinereis vancaurica*

| Peptides | Concentrations (M) $10^{-8}$ | $10^{-7}$ | $10^{-6}$ | $10^{-5}$ |
|---|---|---|---|---|
| Myomodulin-CARP-family peptides | | | | |
| AMGMLRMamide | + | ++ | +++ | NT |
| AMPMLRLamide | NT | − | ++ | +++ |
| PMSMLRLamide | − | + | +++ | NT |
| GSYRMMRLamide | − | + | ++ | +++ |
| PMNMLRLamide | NT | − | ++ | +++ |
| SLGMLRLamide | − | + | ++ | +++ |
| GLNMLRLamide | − | + | ++ | +++ |
| pQLSMLRLamide | − | + | ++ | +++ |
| pQLPMLRLamide | − | − | − | +++ |
| GLHMLRLamide | − | + | ++ | +++ |
| Analogue peptides | | | | |
| AMPMLRMamide | − | ++ | +++ | NT |
| PMSMLRMamide | − | ++ | +++ | NT |
| AMGMLRLamide | NT | − | − | ++ |
| AMPMLRFamide | NT | NT | − | + |
| PMSMLRFamide | − | − | + | ++ |
| AMPMLRL | NT | NT | NT | − |
| MLRLamide | NT | + | ++ | +++ |
| WLRLamide | NT | − | − | +++ |
| LRLamide | NT | − | − | − |

+++, strong effect. ++, moderate effect. +, weak effect. −, no effect. NT, not tested. pQ, pyroglutamic acid.

but Leu-Arg-Leu-$NH_2$ did not show any effect at $10^{-5}$ M or lower. Replacement of the Met residue of Met-Leu-Arg-Leu-$NH_2$ with a more hydrophobic residue Trp decreased the contractile potency.

The effects of Pev-myomodulin on catch tension and phasic contraction of the ABRM were also examined, and compared with those of CARP. The effects of $10^{-6}$ M of these peptides on a preparation of the ABRM are shown in Figure 5. The actions of CARP and Pev-myomodulin ($10^{-6}$ M) on the phasic contractions were apparently opposite. However, those actions were found to be dose-dependent (Fig. 6), indicating that actions of these peptides on the phasic contractions were not qualitatively different. Dose-response relationships of these peptides showed that CARP was much more potent than Pev-myomodulin in exerting both modulatory effects on the phasic contraction and relaxing effects on the catch tension (Fig. 6).

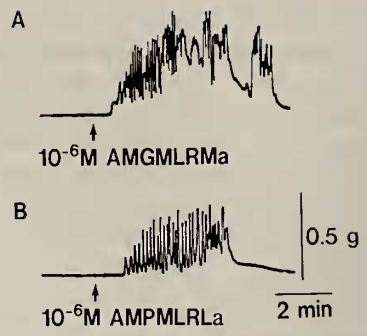


Fig. 4. Actions of $10^{-6}$ M AMGMLRMamide (Pev-myomodulin) and $10^{-6}$ M AMPMLRLamide (CARP) on the isolated esophagus of *P. vancaurica*. A, Pev-myomodulin. B, CARP.

## DISCUSSION

The novel heptapeptide Pev-myomodulin isolated from the annelid, *P. vancaurica*, in the current study is apparently a member of myomodulin-CARP family. The members of the myomodulin-CARP family have so far been identified only in molluscs (Table 1). Thus, this is the first report on

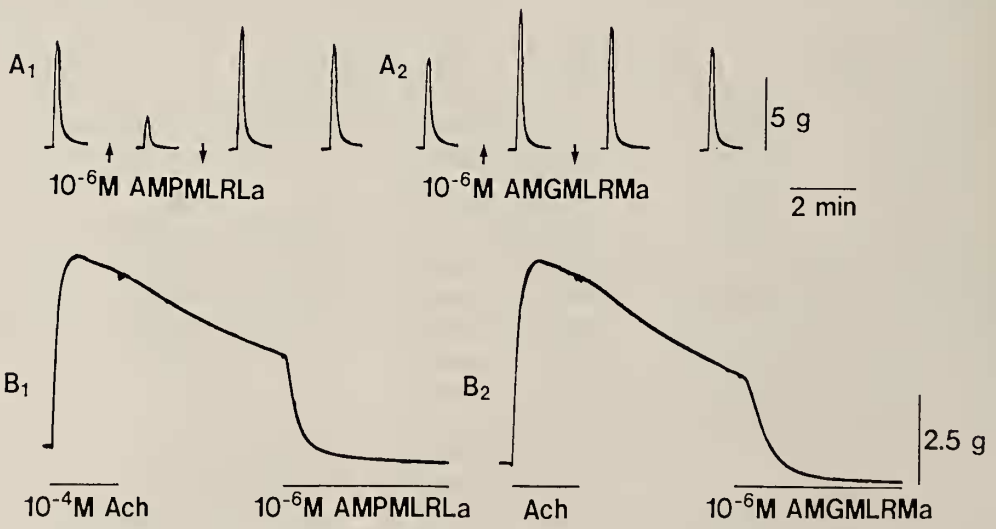


Fig. 5. Effects of AMPMLRLamide (CARP) and AMGMLRMamide (Pev-myomodulin) on phasic contractions ($A_1$, $A_2$) in response to repetitive electrical stimulation (15 V, 3 msec, 10 Hz, 50 pulses) and on catch contractions ($B_1$, $B_2$) induced by $10^{-4}$ M ACh.

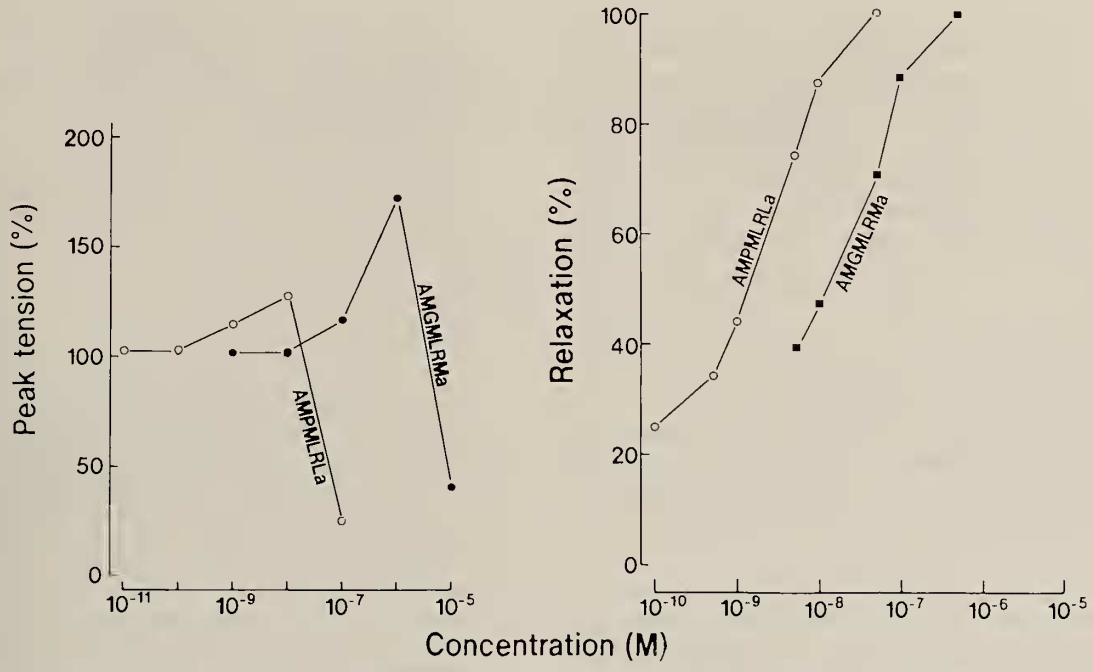


FIG. 6. Dose-response relationships of AMPMLRLamide (CARP) and AMGMLRMamide (Pev-myomodulin) for modulating actions on the phasic contractions (left) and for relaxing actions on the catch contractions (right) of the *Mytilus* ABRM.

the occurrence of a member of the family in the Annelida. Since Pev-myomodulin showed a potent contractile effect on the esophagus of *P. vancaurica,* this peptide may be involved in the regulation of the esophagus of the animal. We have isolated previously two S-Iamide peptides from *P. vancaurica,* both of which elicit spontaneous contraction of the esophagus of the same animal as did Pev-myomodulin [16]. It is probable that some other peptides, which affect gut motility, may be found in *P. vancaurica,* and in other annelids as well, in future.

A conspicuous difference in structure between the annelid peptide and the molluscan peptides is at their C-termini. The annelid peptide has a Met-$NH_2$ at its C-terminus, while all the molluscan peptides have a Leu-$NH_2$. Among the myomodulin-CARP-family peptides including Pev-myomodulin, the annelid peptide shows the most potent contractile effect on the esophagus of *P. vancaurica.* The C-terminal Met-$NH_2$ of the peptide seems to be important in eliciting the potent effect. This notion is supported by the facts that $Leu^7$-Pev-myomodulin is far less potent than Pev-myomodulin and that $Met^7$-CARP and $Met^7$-myomodulin are more potent than CARP and myomodulin, respectively.

The synthetic analogues $Phe^7$-CARP and $Phe^7$-myomodulin show less potent effects than CARP and myomodulin, respectively. This may be partly due to FMRFamide-like actions of the analogue peptides, because it has been known that FMRFamide has an inhibitory action on the esophagus of a polychaete annelid, *N. virens* [14]. FMRFamide has been shown to be present in some annelids [1, 4, 13].

C-terminus-free CARP, Ala-Met-Pro-Met-Leu-Arg-Leu-OH, does not show any contractile effect on the esophagus, suggesting that the C-terminal amide is essential for exertion of the contractile effects of the myomodulin-CARP-family peptides. It has been reported that the C-terminal amide is essential for catch-relaxing action of CARP on the ABRM of *M. edulis* [6]. Another example for essentiality of C-terminal amide for bioactivity is the RPCH-related peptide, APGWamide, which has been isolated from ganglia of the gastropod mollusc *Fusinus ferrugineus* [15]. On electrically-elicited phasic contractions of the *Mytilus* ABRM, the C-terminal dipeptide fragment of APGWamide, GWamide, shows a comparable activity to the native tetrapeptide, while C-terminal-free dipeptide, GW, does not show any activity [17]. Therefore, the C-terminal amide of small bioactive peptides having amidated C-termini may be generally essential for exertion of their bioactivity.

The tetrapeptide fragment, Met-Leu-Arg-Leu-$NH_2$, shows a considerable effect on the esophagus, but the tripeptide fragment, Leu-Arg-Leu-$NH_2$, does not show any effect. These facts suggest that the C-terminal tetrapeptide sequence is the minimum structure required for expression of the contractile effect of the myomodulin-CARP-family peptides on the esophagus, though the Met residue can be substituted at least with Trp.

It has been shown that CARP potentiates phasic contraction of the ABRM of *M. edulis* in response to repetitive electrical stimulation at lower doses and inhibits at higher doses [7, 18]. The annelid peptide Pev-myomodulin also shows a potentiating effect at lower doses and an inhibitory effect at higher doses on phasic contraction of the ABRM. However, CARP exerts these effects at about 100 times lower

concentrations than Pev-myomodulin. As shown in Figure 5A, therefore, $10^{-6}$ M CARP inhibits the phasic contraction while $10^{-6}$ M Pev-myomodulin potentiates it. The reason why the myomodulin-CARP-family peptides show such dual actions is obscure at present. The annelid peptide, as well as CARP, relaxes catch tension of the ABRM. However, the annelid peptide is approximately 10 times less potent than CARP. In the ABRM, therefore, the authentic peptide CARP is more potent than the foreigh peptide Pev-myomodulin.

In conclusion, we isolated a novel member of myomodulin-CARP family from the marine polychaete, *P. vancaurica*. It has been shown that S-Iamide-family peptides [16] and FMRFamide-family peptides [1, 4, 13] are present not only in molluscs but also in annelids. Annelids seem to have many neuropeptides closely related to molluscan neuropeptides.

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